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Human ACAT-1 and -2 inhibitory activities of saucerneol B, manassantin A and B isolated from *Saururus chinensis*

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Abstract—The sesquineolignan, saucerneol B (**1**), and dineolignans, manassantin A (**2**), and manassantin B (**3**), were isolated from the methanol extracts of *Saururus chinensis* root and elucidated by their spectroscopic data analysis. Compounds **1**–**3** inhibited hACAT-1 and hACAT-2 with IC_{50} values of 43.0 and 124.0 μM for **1**, of 39.0 and 8.0 μM for **2**, of 82.0 μM and only 32% inhibition at 1 mM for **3**, respectively. The EtOAc-soluble fraction, which contained compounds **1**–**3**, of methanol extracts of *S. chinensis* exhibited strong cholesterol-lowering effect in high cholesterol-fed mice.

Acyl-CoA: cholesterol acyltransferase (ACAT, E.C. 2.3.1.26) is an allosteric enzyme that catalyzes the acylation of cholesterol to cholesteryl esters with long chain fatty acids.[1] Recently, it was found to be present as two isoforms, ACAT-1 and ACAT-2, with different tissue distribution and membrane topology in mammalian species.[2] In mice and monkeys, ACAT-2 is prominent in liver and intestine, where ACAT-1 is ubiquitous in peripheral tissues. In humans, the major isoform in intestine is ACAT-2, but ACAT-1 is the major isoform in human liver. While both ACAT-1 and ACAT-2 are present in the liver and intestine, the cellular localization of the two enzymes is distinct. In nonhuman primates, ACAT-1 is found primarily in the Kupffer cells of the liver, kidney, and adrenal cortical cells, while ACAT-2 is mainly located in hepatocytes and intestinal mucosal cells.[3] In humans, both ACAT-1 and ACAT-2 are present hepatocytes and HepG2 cells.[3] ACAT-1 plays a critical role in foam cell formation in macrophages; whereas ACAT-2 is in charge of the cholesterol absorption process in intestinal mucosal cells.[4] These findings were consistent with the following results that atherosclerosis lesions were reduced at ACAT-$1^{-/-}$ mice, whereas ACAT-$2^{-/-}$ mice have limited cholesterol absorption in the intestine, and decreased cholesterol ester content in the liver and plasma lipoproteins.[5] Therefore, ACAT-1 and ACAT-2 inhibition is a useful strategy for treating hypercholesterolemia, cholesterol gallstones, or atherosclerosis.

Saururus chinensis Baill (Saururaceae) is a perennial herbaceous plant with potential therapeutic utility in treatment of various diseases such as edema, jaundice, gonorrhea, anti-pyretic, diuretic, and anti-inflammatory agent.[6] These neolignan series, which are tetrahydrofuran type, are known to have a variety of biological activities, such as cell adhesion inhibitory,[7] anti-inflammatory,[8] anti-plasmodial,[9] and murine neuroleptic[10] activities. In connection of our studies on the isolation and structure elucidation of potential antioxidant from *S. chinensis* plants,[11] we found the methanol extracts of *S. chinensis* exhibited significant hACAT inhibitory activity (79% inhibition for hACAT-1, 53% inhibition for hACAT-2 at 100 μg/mL, respectively). Subsequent bioactivity-guided fractionation of the methanol

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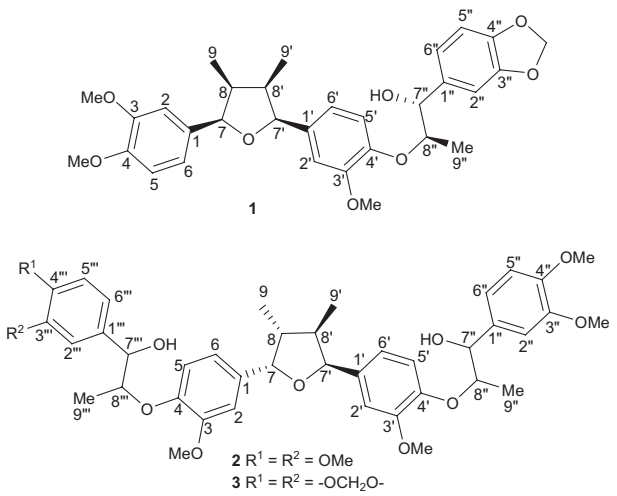

Figure 1. Chemical structures of **1**–**3** isolated from *S. chinensis*.

extracts led to the sesquineolignan, saucerneol B (**1**), and dineolignans, manassantin A (**2**), and manassantin B (**3**). Recently, we reported mass production of human ACAT-1 and ACAT-2 individually from Hi5 cells, which was infected by recombinant baculoviruses to screen isoform-specific inhibitor.[12] In this study, we describe the isolation, structure characterization, and hACAT-1 and hACAT-2 inhibitory activities of three compounds **1**–**3** (Fig. 1).

The dried roots of *S. chinensis* (500 g), which were collected on Geochang, Korea, were ground and extracted with 95% MeOH (1.5 L × 2) at room temperature for 24 h. After filtration and concentration, the MeOH extract (75 g) was suspended in H_2O and then partitioned with the order of *n*-hexane, $CHCl_3$, and EtOAc to give three fractions, *n*-hexane- (9.0 g), $CHCl_3$- (17.0 g), and EtOAc-soluble residue (22.0 g), respectively. The EtOAc-soluble fraction exhibited activities against hACAT-1 and hACAT-2, with 81% and 58% inhibition at 100 μg/mL, was subjected to flash silica gel column chromatography with a gradient of chloroform–methanol (100:0 to 0:100, v/v) to give five fractions. By bioassay-guided fractionation, the one of active fractions was purified by column chromatography on silica gel with a gradient of *n*-hexane–ethyl acetate (3:1 to 2:1, v/v) to obtain the sesquineolignan, saucerneol B (**1**) (20 mg). Subsequently, the other active fraction was subjected to column chromatography on silica gel with a gradient of *n*-hexane–ethyl acetate (1:1 to 1:2, v/v) to give dineolignans, manassantin A (**2**) (200 mg), and manassantin B (**3**) (1 g).

Compound **1**, an amorphous powder, showed the value of $[\alpha]_D^{25}$ −51.0 (*c* 0.6, $CHCl_3$) (lit. $[\alpha]_D^{25}$ −58.0 (*c* 0.6, $CHCl_3$)) and exhibited λ_{max} at 233 ($\log\varepsilon$: 4.22) and 283 ($\log\varepsilon$: 4.22) (MeOH).[13] A molecular formula of $C_{31}H_{36}O_8$ was determined by HRFABMS data (*m*/*z* $[M]^+$, 536.2408, calcd 536.2410 for $C_{31}H_{36}O_8$). The IR spectrum of **1** showed strong hydroxyl absorption bands at 3481 cm^{-1} due to hydroxy group. The ^1H NMR spectrum of **1** showed singlets at δ_H 3.87, 3.89, 3.92, and 5.95 corresponding to 11H that were attributed to three methoxy and methylenedioxy groups. Three aromatic signals were displayed between δ_H 6.76 and 6.99 (9H, m). Additionally, doublet at δ_H 0.70 (6H, d, $J = 4.8$ Hz, H-9, 9″) of the methyl groups at C-8 and C-8′ with four methine groups at δ_H 2.27 (2H, m, H-8, H-8′) and 5.42 (2H, t like, $J = 6.0$ Hz, H-7, H-7′) pointed to tetrahydrofuran moiety. Also, the relative stereochemistry of the tetrahydrofuran ring could be determined by comparing the data reported in the literature.[13]

Compound **2** exhibited a molecular formula of $C_{42}H_{52}O_{11}$ from its positive HRFABMS data (*m*/*z* $[M]^+$, 732.3506, calcd 732.3510 for $C_{42}H_{52}O_{11}$). It has mp 57–58 °C (CH_2Cl_2) and the value of $[\alpha]_D^{25}$ −118.0 (*c* 1.0, $CHCl_3$) (lit. $[\alpha]_D^{25}$ −100.0).[14] Compound **3** demonstrated a molecular formula of $C_{41}H_{48}O_{11}$ by HREIMS data (*m*/*z* $[M]^+$, 716.3198, calcd 716.3197 for $C_{41}H_{48}O_{11}$) and has an mp of 57–58 °C (CH_2Cl_2), and the value of $[\alpha]_D^{25}$ −120.0 (*c* 1.0, $CHCl_3$) (lit. $[\alpha]_D^{25}$ −99.0).[14] The UV spectrum of **2** and **3** exhibited λ_{max} at 233 ($\log\varepsilon$: 4.01) and 280 ($\log\varepsilon$: 3.52) (MeOH) and λ_{max} at 234 ($\log\varepsilon$: 4.40) and 282 ($\log\varepsilon$: 4.04) (MeOH), respectively. The IR spectra of **2** and **3** showed strong absorption bands at 3481 and 3477 cm^{-1} due to two hydroxy groups. All structures of **1**–**3** were determined by spectroscopic data, involving ^1H, ^{13}C NMR, ^1H–^1H COSY, HMQC, and HMBC. The structures of three compounds **1**–**3** were elucidated to be saucerneol B (**1**), manassantin A (**2**), and manassantin B (**3**), comparing their spectroscopic data and previously reported data.[13,14]

Although compounds **1**–**3** have reported to show various biological activities,[6–10] their potential as inhibitor of hACAT-1 or -2 was first time evaluated. The rate of incorporation of [1-^{14}C] oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 or -2.[15] With expecting to show different inhibitor specificity against hACAT-1 and hACAT-2, compounds **1**–**3** were applied to the enzyme assay employing each human ACAT isoform. Saucerneol B (**1**) inhibited hACAT-1 with 3-fold greater activity ($IC_{50} = 43.0$ μM) compared to hACAT-2 ($IC_{50} = 124.0$ μM). Manassantin A (**2**) showed more potent inhibitory activity against hACAT-2 with $IC_{50} = 8.0$ μM compared to hACAT-1 ($IC_{50} = 39.0$ μM). However, manassantin B (**3**) dominantly inhibited hACAT-1 not hACAT-2; the IC_{50} for hACAT-1 was 82 μM while hACAT-2 was inhibited only 32% at 1 mM of manassantin B (**3**). The expressed each isoform and inhibitory activities of the compounds **1**–**3** were confirmed by the positive control with oleic acid anilide, which inhibited hACAT-1 and hACAT-2 with IC_{50} values of 0.14 and 0.17 μM, respectively.[12]

For the development of a useful hypercholesterolemic and anti-atherogenic functional food or agent having ACAT inhibitory activity, we have examined firstly the preliminary cholesterol-lowering activity of the EtOAc-soluble fraction, which contained compounds **1**–**3**, of methanol extract of *S. chinensis* in high cholesterol-fed C59BL/6J mice. The plasma total cholesterol levels were measured after feeding a high cholesterol diet supplemented with 0.5% (wt/wt in diet) of the EtOAc-soluble fraction of *S. chinensis* for 10 days.[16] The EtOAc-soluble

Table 1. The effect of EtOAc layer of *S. chinensis* on plasma total cholesterols of in high cholesterol-fed mice

Group	*N*	Body weight (g)[a]		Total cholesterol (mg/dL)[b]	
		0 day	10 day	0 day	10 day
Control	10	21.8 ± 0.7	23.7 ± 1.1	97.4 ± 8.4	212.6 ± 31.3
EtOAc layer of *S. chinensis* (0.5% wt/wt diet)	10	20.8 ± 0.9	23.1 ± 1.4	101.4 ± 7.2	183.3 ± 25.3*
Probucol (0.1% wt/wt diet)	10	20.9 ± 0.7	23.9 ± 1.2	92.8 ± 13.1	181.9 ± 32.4*

* Significantly different ($p < 0.05$) from control group.
[a] All values are expressed as mean ± SD.
[b] Mean ± SD.

fraction of *S. chinensis* exhibited strong cholesterol-lowering effect (−13.8%) in high cholesterol-fed mice (Table 1). Low dose (0.1% wt/wt in diet) probucol, a known hypocholesterolemic agent, showed a similar degree of hypocholesterolemic activity (−14.4%) as compared to those on same experimental conditions.

Saucerneol B (**1**) preferentially inhibited hACAT-1 than hACAT-2, however manassantin A (**2**) strongly inhibited hACAT-2 compared to hACAT-1. In contrast, manassantin B (**3**) dominantly inhibited hACAT-1 not hACAT-2. In recent studies, pyripyropene A, a known ACAT inhibitor, was identified that specifically inhibits ACAT-2.[12,19] The selective inhibition of ACAT-2 was confirmed by comparing the responses in microsomes isolated from liver samples from ACAT-$1^{-/-}$, ACAT-$2^{-/-}$, and wild-type mice or cells stably expressing hACAT-1 or -2. Lada et al. demonstrated that selective inhibition of ACAT-1 and ACAT-2 provides evidence of uniqueness in structure and function of these two enzymes.[19] Further studies on confirmation of the inhibitory specificity of ACAT-1 or -2 using similar methods described upper and the efficacy test of long term cholesterol-lowering and anti-atherogenic activities of compounds **1**–**3** are underway.

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15. ACAT activity assay: Microsomal fractions of Hi5 cells containing baculovirally expressed hACAT-1 or -2 were used as a source of the enzyme.[12] The activity of the hACAT-1 and hACAT-2 was measured according to the method of Brecher and Chan[17] with slight modification.[18] The reaction mixture, containing 4 μL of microsomes (8 mg/mL protein), 20 μL of 0.5 M potassium–phosphate buffer (pH 7.4) with 10 mM dithiothreitol, 15 μL of bovine serum albumin (fatty acid free, 40 mg/mL), 2 μL of cholesterol in acetone (20 μg/mL, added last), 41 μL of water, and 10 μL of test sample in a total volume of 92 μL, was preincubated for 20 min at 37 °C with brief vortexing and sonication. The reaction was initiated by the addition of 8 μL of [1-^{14}C] oleoyl-CoA solution (0.05 μCi, final concn 10 μM). After 25 min of incubation at 37 °C, the reaction was stopped by the addition of 1.0 mL of

isopropanol–heptane (4:1; v/v) solution. A mixture of 0.6 mL of heptane and 0.4 mL of 0.1 M potassium-phosphate buffer (pH 7.4) with 2 mM dithiothreitol was then added to the terminated reaction mixture. The above solution was mixed and allowed to phase separation under gravity for 2 min. Cholesterol oleate was recovered in the upper heptane phase (total volume 0.9–1.0 mL). The radioactivity in 100 μL of the upper phase was measured in a 3 mL liquid scintillation viral with 3 mL of scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (1450 Micerobeta Trilux Wallac Oy, Turku, Finland). Background values were obtained by preparing heat inactivated microsomes or normal insect cell lysate microsomes, usually background value was 200–250 cpm, while 8000 cpm of ACAT reaction. The ACAT activity was expressed as a defined unit, cholesteryl oleate pmol/min/mg protein.

16. The hypocholesterolemic effect of the EtOAc-soluble fraction of MeOH extract of *S. chinensis* was investigated in male C57BL/6J mice maintained at Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The mice were housed in a room with controlled temperature (22 ± 2 °C), relative humidity (55 ± 5%), and lighting (alternating 12 h cycle of light and dark). At 7 weeks of age, 30 male mice were randomly divided into three groups of 10 animals and fed on a high cholesterol diet (CRF-1 supplemented with 15% fat, 1.25% cholesterol, and 0.5% Na-cholate, Oriental Yeast Co. Ltd, Japan), the first group without supplementation (control), the second group supplemented with 0.5% (wt/wt diet) of the EtOAc-soluble fraction of *S. chinensis*, and third group supplemented with 0.1% (wt/wt diet) of probucol (positive control). Probucol was purchased from Sigma Chemical Co. Ltd. The diet and water were given ad libitum. After treating the test compounds for 10 days, the mice were anesthetized with ethyl ether, and the blood was obtained from the retro-orbital sinus using a heparinized capillary tube. Then, the blood was centrifuged at 8000*g* for 10 min, and the plasma was collected. The concentration of plasma total cholesterol was measured with an automatic blood chemical analyzer (Hitachi 7020, Japan). To evaluate statistical significance between control and experimental groups, student's *t*-test was performed, and a p value of <0.05 was considered to be statistically significant.
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